# BIOTIN DERIVATIVES

## Field of the Invention

The present invention refers to a method for the
conditioning of an extracorporeal device and to a method
for extracorporeal extraction of toxic material from
mammalian body fluids in connection with diagnosis or
treatment of a mammalian condition or disease, in which
methods reagents having the ability to extract toxic
material from mammalian body fluids are involved, and to
an extracorporeal device comprising said reagent.

## Background of the Invention

Toxic materials may be introduced into the blood of
humans by accidents, from disease states, from bacterial
or viral infections, or from administration of substances
for treatment of certain diseases (e.g. cancer therapy).
Many of these toxic materials may do considerable damage
to body tissues such as kidney, liver, lung and bone
marrow, and may even be fatal. It is desirable to remove
such materials from the blood as quickly as possible.
Although the body has natural defense mechanisms to re-
move unwanted toxic materials, those methods can be in-
effective in many examples. Thus, certain toxic materials
are best removed from the blood in an extracorporeal
device. An example of such a device is the kidney dia-
lysis machine, where toxic materials build up in the
blood due to a lack of kidney function. Other medical
applications where an extracorporeal device can be used
include: [1] removal of radioactive materials, [2] re-
moval of toxic levels of metals, [3] removal of toxins
produced from bacteria or viruses, [4] removal of toxic
levels of drugs, and [5] removal of whole cells (e.g.

cancerous cells, specific hematopoietic cells - e.g. B,
T, or NK cells) or removal of bacteria and viruses.

In order for the extracorporeal device to function
in toxin removal, it must have a chemical entity bound on
5   it that has a high binding affinity with the toxic mater-
ial that is to be removed from blood. Rather than binding
that chemical entity directly to the column matrix in the
extracorporeal device, it is preferentially bound through
another binding pair of molecules. This arrangement of
10  binding is used to make the toxin binding moiety more
available in the blood and to make the device more gene-
rally applicable to a variety of toxic materials. A
column matrix material is used that provides a high sur-
face area while not restricting the flow of blood through
15  it (Nilson, R. et.al. EPC 567 514). The column matrix has
a protein (avidin or streptavidin) bound to it that has a
high affinity for another molecule (e.g. biotin). That
column is conditioned for use in a particular medical
application by conjugation of a moiety that has a high
20  affinity for the toxic material with two molecules of
biotin such that attachment to the column matrix can be
readily achieved. This conditioning reagent contains two
biotin moieties rather than one as this configuration
provides a higher degree of stability to the column mat-
25  rix.

Although, tumor-specific immunoconjugates are selec-
tively bound to tumor cells, an initial high concentra-
tion of the cell-toxic immunoconjugate in the blood cir-
culation is necessary to reach a sufficient high concen-
30  tration of the target tissue in a patient. While required
for optimal therapy of the cancer, the high concentration
of cytotoxic material in the blood and other non-tumor
tissues, in most cases leads to tissue damage and/or

lesion formation in sensitive and vital tissues like the bone marrow. Although, bone marrow rescue is sometimes used to circumvent these potentially lethal effects, such transplantation is both extremely costly and possesses a

5 high risk for the patient. Even in cases where the bone marrow transplantation is effective, other sensitive organs like the, liver, kidney, spleen, lung etc. can be irreparably damaged. The most effective method for preventing tissue and bone marrow damage from toxic mater-

10 ials in blood is to dramatically decrease the amount of that toxic material in the blood. Of course, this must be accomplished in a manner that retains the therapeutic level of toxic material in the tissue being treated (e.g. tumor).

15    Radiolabeled antibodies have been under investigation for therapy of cancer for several decades. Administration of radiolabeled antibodies introduces a toxic material into blood. Various methods have been proposed to rapidly clear radiolabeled antibodies from blood

20 circulation after the tumor has accumulated a sufficient quantity of immunoconjugate to obtain a diagnosis or therapy. Some of the methods employed involve enhancement of the bodies own clearing mechanism through the formation of immune complexes. Enhanced blood clearance of

25 radiolabeled antibodies can be obtained by using molecules that bind it, such as other monoclonal antibodies (Klibanov et. al., J. Nucl. Med. 29, 1951-1956, 1988; Marshall et al. Br. J. Cancer 69, 502-507, 1994; Sharkey et al. Bioconjugate Chem. 8, 595-604, 1997), avidin/-

30 streptavidin (Sinitsyn et al., J. Nucl. Med. 30, 66-69,1989; Marshall et. al., Br. J. Cancer, 71, 18-24, 1995), or glycosyl containing compounds which are removed by receptors on liver cells (Ashwell and Morell, Adv.

Enzymol. 41, 99-128, 1974). Still other methods involve removing the circulating immunoconjugates through extra-corporeal methods (see review article by Schriber, G.J. & Kerr, D.E., Current Medical Chemistry, 1995, Vol. 2, pp

5   616-629).

The extracorporeal techniques used to clear a medical agent from blood circulation are particularly attractive because the toxic material is rapidly removed from the body. Application of these methods in the context of

10  immunotherapy have been previously described (Henry CA, 1991, Vol.18, pp. 565; Hofheinze D et al., Proc. Am. Assoc. Cancer. Res. 1987 Vol. 28, pp 391; Lear J K, et al. Radiology 1991, Vol. 179, pp. 509-512; Johnson T.K. et. al. Antibody Immunoconj. Radiopharm. 1991, Vol. 4,

15  pp. 509; Dienhart D.G., et al. Antibody Immunoconj. Radiopharm. 1991, Vol. 7, pp. 225; DeNardo G.L. et al. J. Nucl. Med. 1993, Vol. 34, pp 1020-1027; DeNardo S.J. et. al. J. Nucl. Med. 1992, Vol. 33, pp. 862-863; DeNardo G.L. J. Nucl. Med. 1992, Vol.33, pp. 863-864; and U.S.

20  patent Number 5,474, 772; Australian Patent 638061, EPO; and EPO 90 914303.4 of Maddock.

To make the blood clearance more efficient and to enable processing of whole blood, rather than blood plasma as the above methods refer to, the medical agents

25  ( e.g. tumor specific monoclonal antibody carrying cell killing agents or radionuclides for tumor localization) have been biotinylated and cleared by an avidin based adsorbent on a column matrix. A number of publications provide data showing that this technique is both effici-

30  ent and practical for the clearance of biotinylated and radionuclide labeled tumor specific antibodies (Norrgren K, et. al. Antibody Immunoconj. Radiopharm. 1991, Vol. 4, pp 54; Norrgren K, et .al. J. Nucl. Med. 1993, Vol. 34,

pp. 448-454; Garkavij M, et. al. Acta Oncologica 1996,
Vol. 53, pp.309-312; Garkavij M, et. al. J. Nucl. Med.
1997, Vol.38, pp.895-901. These techniques are also de-
scribed in U.S. Patent application No. 08/090,047;

5    EPC 567 514 and 08/434,889).

     Apart from the prolonged circulation time leading to
undesired exposure of toxic immunoconjugate to healthy
tissue, inadequate tumor tissue penetration and non-
specific organ retention and metabolism contribute to a

10   low therapeutic index ratio. Due to these problems,
multi-step antibody-based radionuclide delivery approach-
es have been extensively investigated. The basic concept
involves first the injection of a lesion-specific tar-
geting moiety which apart from binding specifically to

15   the lesion also has the feature of binding to a subse-
quently injected radioactive diagnostic agent or a thera-
peutic agent. By separating these two events one can
allow the slow tissue penetrating non-radioactive/non-
cytotoxic antibody sufficient time to accumulate in the

20   tumor mass, while the agent carrying the radionuclide/-
cytotoxin could be selected for more rapid tissue pene-
tration. However, a prerequisite is that the former (and
preferably also the later) can be cleared rapidly from
the blood circulation.

25    Most of these multi-step approaches utilize binding
pairs of avidin/streptavidin and biotin. Avidin is a 67
kDa glycoprotein found in egg whites and tissue of birds
and amphibia. It consists of 4 non-covalently bound
subunits. Each subunit is capable of binding one biotin

30   molecule. Avidin has a high isoelectric point ( pI>10),
due to its 36 lysine amino acid residues, which results
in non-specific binding to cellular membranes. Strept-
avidin (SAv), produced in Streptomyces avidinii, is a

close relative of avidin. It shares high affinity to
biotin, but differs in amino acid content as well as net
charge (pI 6.5) and is not glycosylated. Due to lack of
sugar groups, SAv has a slightly lower molecular weight

5    of 60 kDa and the in vivo pharmacokinetics and biodistri-
bution differs markedly from avidin. Whereas intravenous
injection of radiolabelled avidin clears rapidly from the
blood and accumulates extensively in the liver, radio-
labeled SAv exhibits a much longer circulation time, and

10   has lower organ accumulation (Pimm MV et al. Nucl. Med.
Comm.1988 Vol. 9, 931-941; Schechter B et al., Eur. J.
Biochem. 1990, Vol. 189, 327-331; Rosebrough SF, Nucl.
Med. Biol. 1993, Vol.20, 663-668).

The other part of the binding pair, biotin, is a

15   vitamin and a member of the B-complex, which is essential
for amino acid and odd-chain fatty acid synthesis. Biotin
in found preferentially intracellular, usually bound to
an enzyme and acts as a co-factor during carboxylation
reactions. Biotin is often present as a lysine-biotin

20   adduct (biocytin), in food and during metabolic protein
turnover. The linkage between lysine and biotin is
cleaved by a plasma enzyme, biotinidase.

To improve the imaging in patients with carcinoma of
the lung, Kalofonos et. al. used a two-step  SAv-MAb /

25   111In DTPA-biotin approach (Kalofonos HP et al., J. Nucl.
Med. 1990, Vol. 31, 1791-1796). Van Osdol et. Al. And
Sung et. al. have developed a mathematical model of two-
step imaging, and treatment protocols using SAv-MAb and
radiolabelled biotin chelates. Taken into account the in

30   vivo parameters of both the targeting SAv-MAb moiety and
the radiolabelled biotin imaging agent, they predicted
the following:

1)    The large molecular weight of SAv-MAb will

reduce the amount of MAb that will localize in the tumor and the binding homogeneity in the tumor.

2)   Radiolabelled biotin will diffuse rapidly into the tumor, but due to the high affinity to peripheral

5   tumor-bound SAv-Mab, will not penetrate deeply into the nodule  if too low dose is given.

3)   Compared to directly labeled MAbs, the two-step SAv-MAb/ radiolabelled biotin protocol permits imaging sooner after radioactive injection and produced higher

10   tumor/blood ratios.

4)   That tumor/blood ratios at 24 hrs are > 2 times higher that with the use of directly labeled MAbs.

In their simulation, a high percentage of the radio-activity is bound to circulating SAv-MAb and that the

15   addition of clearing agent before radiolabelled biotin was injected would enhance the tumor blood ratio.

A two-step approach using biotinylated MAbs and radiolabelled SAv has also been utilized in animal models as well as in patients (Paganelli G. et. al. Eur. J.

20   Nucl. Med. 1992, Vol. 19, 322-329; Khawli LA et al. Abs. Immunoconj. Radiopharm. 1993 Vol. 6, 13-27; Kassis AI. et. al. J. Nucl. Med. 1996 Vol. 35, 1358-1365). In this method, both the targeting and the imaging agents are of large molecular weight and clear slowly from the blood.

25   The whole procedure takes many days to complete and with metabolism, radioactivity accumulates in organs and is slowly eliminated from the body. Nevertheless, these studies showed that biotin/SAv binding was accomplished in vivo and yielded positive images and enhanced tumor

30   activity compared to directly labeled MAbs.

A three-step procedure consisting of biotinylated MAb, avidin and then followed by 111In-DTPA-biotin has also been tried (Paganelli G et al. Canc. Res. 1991 Vol.

51, 5960-5966; Dosio F. et. al. J. Nucl. Biol. Med. 1993
Vol.37, 228-232). This procedure required 1-3 days be-
tween injections to allow for tumor accumulation and
blood clearance. As a whole, all these studies have shown

5    the feasibility of immunological approaches utilizing the
SAv / biotin system in vivo. However, circulating levels
of the high molecular weight targeting agents were pro-
blematic due to their prolonged circulation and non-
specific organ accumulation.

10        An alternative pretargeting approach uses three
separate injections of three components: [1] SAv-Mab,
[2] a clearing agent, and [3] a radiolabelled biotin
derivative containing the radiometal chelation moiety
DOTA has been thoroughly investigated (Axworthy DB et al.

15    J. Immunother. 1994, Vol. 16, 158). A covalent conjugate
of tumor-specific MAb and SAv is injected and is allowed
to accumulate at tumor sites. After sufficient tumor
uptake (24-48 hrs) a biotin clearing agent is administer-
ed in order to clear the blood from the conjugate through

20    the liver. Finally, the radiolabelled biotin-DOTA deri-
vative is injected. The clearing agent used in this con-
text is typically a biotinylated protein to which galac-
tose residues have been conjugated. The galactose recep-
tors resides on hepatocytes and exhibit a high affinity

25    and specificity for macromolecules with exposed terminal
galactose residues. The hepatic uptake correlate with the
amount of galactose residues bound to SAv (Rosebrough SF,
J. Nucl. Med. 1996, Vol.37, 344-350).

       In all these concepts there is bound to be a con-
30    flict between initial concentration of the targeting
molecule and its ability to penetrate deep into the tumor
on one hand, and a rapid and complete clearance from the
blood prior to administration of the radioactive/cyto-

toxic agent, on the other. In principle, the same condition applies for the radioactive/cytotoxic agent. A sufficiently high initial blood concentration is essential to reach and saturate the targeting molecule. At the

5 same time this toxic agent must not reside in the blood circulation and exposing sensitive tissues like the bone marrow. Even if the toxic agent is cleared fairly rapidly through the body, organs like the kidney and the urinary track will normally receive an accumulated toxic dose

10 equally or higher to that received by the tumor tissue.

There is clearly a need for optimizing these and other therapy protocol conditions, particularly if the approaches are going to be adequate for the treatment of solid tumors. It is vital that such concepts are to a

15 large extent generic, in so far that as many as possible of the parameters are independent on the type and localization of the disease and as much as possible independent on the pharmacokinetic parameters and rate of metabolisms of the individual patient.

20 Summary of the Invention

The object of the present invention is to eliminate the above-mentioined problems with toxic or undesired compounds in body fluids. This object is achieved with a method according to the present invention for the condi-

25 tioning of an extracorporeal device and a method for the extracorporeal extraction of toxic material from mammalian body fluids in connection with diagnosis or treatment of a mammalian condition or disease, in which methods a reagent is used having the ability to extract

30 toxic material from body fluids and comprising the general formula:

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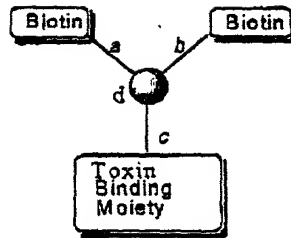
20     wherein the biotin moieties are natural biotin or
derivatives thereof,

    wherein a, b, and c are linkers, which are same or
different, and wherein d is a trifunctional crosslinking
moiety, said reagent is alternately called conditioning
25 reagent in the description, or with preferred embodiments
of said methods, in which the toxin binding moiety of the
reagent is biotin or a derivative thereof for the extra-
corporeal extraction of toxic material from mammalian
body fluids in connection with diagnosis or treatment of
30 a mammalian condition or disease. Other objects and
advantages will become apparent from the detailed
description of the invention and the appended subclaims.

    In one aspect of the present invention, reactive
dibiotin compounds are coupled with ligands that selec-
35 tively bind with components naturally found in, or
artificially introduced into, blood, and the resulting
conjugates are used to condition avidin or streptavidin
containing column matrixes for medical applications. In
another aspect of the present invention, an extracorpore-
40 al device containing a column that has been conditioned
with the dibiotin conjugate is connected to an apparatus
that pumps whole blood from patients through the column

and back into the patient to cleanse the patients blood
of materials that bind with the dibiotin conjugate. Thus,
the objective of the present invention is also to facili-
tate the use of extracorporeal clearance of toxic agents
5     by providing means of a one step conversion of a biotin-
binding device to toxic substance binding device by the
use of water soluble molecule that contains two biotin
moieties.

Other aspects, wherein the reagent is tribiotinylat-
10    ed, are described in the following examples.

Short description of the Drawings

Fig. 1 illustrates the conditioning of a (strept)avidin
       matrix with derivatized dibiotin compounds.

Fig. 2 illustrates the binding of toxic material with a
15     conditioned column.

Fig. 3 illustrates the generic structure of the column
       conditioning reagent.

Fig. 4 shows a standard curve for biotin-trimer ELISA.

Fig. 5 shows a typical depletion curve of avidin by an
20     biotin-trimer/avidin column.

Disclosure of Preferred Embodiments

The present invention also involves a conditioning
method for converting a biotin-binding matrix to a matrix
that can bind a variety of substances toxic to the body.
25    Specialized avidin or SAv coated columns are used in the
invention as these provide a binding surface for toxic
compounds (e.g. radiolabeled antibodies) which have
biotin attached (Nilsson. R. et.al. ,EPC 567 514). These
columns have the proper characteristics for passing whole
30    blood over them and obtaining good clearance in a
reasonable time period. The present invention greatly
expands the use of the avidin/SAv columns by their
conversion to bind other types of molecules in a two step

process. In the first step, column conversion from the biotin-binding (strept)avidin coated column to a column that binds a toxic material can be accomplished by conditioning it with an excess of a dibiotin derivative

5    as shown in Figure 1. In a second step, the conditioned column can be used in an extracorporeal device to rid the blood of the toxic substance. Binding of the toxic substance with the conditioned column is depicted in Figure 2. The two steps can be separated by time with

10   storage of the conditioned column.

In a preferred embodiment of the invention, a toxic medical agent used for therapy of human disease is removed from the blood to improve its ratio of target-to-non-target concentration. An improved target-to-non-

15   target ratio provides a better therapeutic index. Specific tissue or organ localization of a medical agent is a very important factor in its effective application. Lack of specific tissue localization is of particular importance in the treatment with medical agents, where the de-

20   sired effect is to kill certain types of cells such as in the treatment of cancer. In order to enhance the specificity, tumor specific monoclonal antibodies are used as a carrier (immunoconjugates) of various cytotoxic agents, such as, but not limited to, radionuclides, cytotoxins,

25   and enzymes used in pro-drug protocols ( Meyer et al., Bioconjugate Chem. 6, 440-446; 1995; Houba et al., Bioconjugate Chem. 7, 606-611, 1996; Blakey et al., Cancer Res. 56, 3287 -3292, 1996).

The term "reagent" (also "conditioning reagent")

30   used herein means a compound containing two functions for specific interaction with a single biotin binding molecule and a separate function for the binding of toxic material. It can be used to convert a biotin binding

matrix to a matrix that can be used to extract specific toxic material from body fluids or be used to convert a biotin binding matrix to a matrix which binds biotin binding molecules.

5     The term "effector molecule" used herein means any moiety which can be linked to a targeting molecule or to a molecule interacting with a targeting molecule (targeting molecule complex) and which either enhances the effect of the targeting molecule/ targeting molecule

10    complex or which alone contribute to a desirable pharmacological or diagnostic effect.

    The term "toxic material" used herein means any compound, optionally in a conjugate with an effector molecule, cluster of compounds, cells etc. which can be

15    extracted from mammalian body fluids by the use of the reagent.

    The term "toxic binding moiety" used herein means any moiety capable of extracting toxic material from mammalian body fluid by specifically interacting with the

20    toxic material. In some preferred embodiments of the present invention the toxic binding moiety is biotin or a derivative thereof.

    The term "biotinylated molecules" used herein means molecules that penetrates mammalian tissue more readily

25    than immunoglobulines, e.g. a radiolabelled biotin derivative containing a radiometal chelation moiety.

    The term "targeting biomolecule" used herein means biomolecules which selectively bind to certain structures on mammalian cells.

30    For affinity adsorbents the matrix (M) may be of various shape and chemical composition. It may for example constitute a column house filled with particulate polymers, the latter of natural origin or artificially

made. The particles may be macroporous or their surface
may be grafted, the latter in order to enlarge the sur-
face area. The particles may be spherical or granulated
and be based on polysaccharides, ceramic material, glass,
5  silica, plastic, or any combination of these or alike
material. A combination of these could for example be
solid particles coated with a suitable polymer of natural
origin or artificially made. Artificial membranes may
also be used. These may be flat sheet membranes made of
10  cellulose, polyamide, polysulfone, polypropylene or other
types of material which are sufficiently inert, biocompa-
tible, non-toxic and to which the receptor could be
immobilized either directly or after chemical modifica-
tion of the membrane surface. Capillary membranes like
15  the hollow fibers made from cellulose, polypropylene or
other materials suitable for this type of membranes may
also be used. A preferred embodiment is a particulate
material based on agarose and suitable for extracorporeal
applications.
20      The biotin-binding molecule may be immobilized to
matrix by various methods. The coupling method of choice
will depend on the nature of the biotin-binding molecule
as well as the nature of the support matrix. For pro-
teins, functional groups such as hydroxyl-, amino-,
25  carboxyl- or thiolgroups may be utilized. Glycoproteins
like avidin may be coupled to the matrix via their glyco-
syl residues. The solid support may also be activated to
enable binding of proteins by forming linkages with the
solid support through specific or non-specific reaction
30  with the side-chains or the backbone structure of the
protein. The linkage between the solid support and the
biotin-binding molecule may also be of non-covalent
nature, where electrostatic, hydrogen bonding or hydro-

phobic forces are utilized. For applications in immuno-
assays a non-covalent attachment would be most appro-
priate. A spacer between the matrix and the biotin-bind-
ing molecule may also be used.

5       The conditioning reagent is composed of a molecule
that has two biotin moieties as well as a toxic material
binding moiety. Two biotin moieties are important in the
invention as this arrangement allows both biotin moieties
to bind with the same (strept)avidin molecule on the

10   column, which cross-links the subunits and helps to
stabilize the column to the conditions of blood movement
through it. Cross-linking of biotin moieties with adja-
cent (strept)avidin moieties does not occur as the biotin
dimers bind much more rapidly with the adjacent biotin

15   binding pocket (intramolecular binding) rather than with
a biotin binding pocket on an adjacent molecule (inter-
molecular binding) [Wilbur et al. Bioconjugate Chem. 8,
819-832, 1997). Avidin and streptavidin are more stable
when biotin is bound, and cross-linking the subunits

20   provides additional stability ( Biomolecular Engineering
16, 67-72, 1999 ).

The conditioning reagent is comprised of two biotin
moieties, a toxin binding moiety, a trifunctional cross-
linking moiety, and three linker molecules (a-c) as

25   depicted in Figure 3. The two biotin moieties are com-
prised of natural biotin or biotin derivatives coupled to
the linker molecule(s) (a,b) through the biotin carboxy-
late via an amide bond. The biotin moieties are coupled
to the trifunctional cross-linking reagent through linker

30   molecules that provide a minimum of 20 Å and a maximum of
60 Å between the biotin carboxylate carbon atoms when
measured in a fully linearized form. Linker molecules a,
b and c, also called-linkers for short, may be the same

or each linker may be of a different nature. The linker
molecules are linear or branched chained and contain
water solubilizing functionalities such as ether/-
thioether bonds, amines, in the chain, or appendages to

5   the chain which contain amines, carboxylates or hydroxyl
functionalities. The atom on the linker alpha to the
biotinamide bond may be unsubstituted (e.g. $CH_2$) or may
contain a methyl, hydroxymethylene, or carboxylate func-
tionality. Larger functionalities diminish the binding

10   with (strept)avidin columns. The later functionalities
provide stability from biotinidase, but this stability is
not required for the two biotin moieties binding with
(strept)avidin on the columns as they are not available
for cleavage by biotinidase. When the toxin binding

15   moiety is another biotin moiety, the biotinidase sta-
bility may be desired. The trifunctional cross-linking
reagent is an aliphatic or aromatic compound that con-
tains three functional groups that are nucleophiles, or
are reactive with nucleophiles, for conjugation with the

20   linker molecules. Preferred trifunctional cross-linking
reagents are aromatic rings with 1,3,5-substitution.
Most preferred are derivatives of 1,3,5-benzene tricar-
boxylic acid, 3,5-diaminobenzoic acid, 5-amino-1,3-di-
carboxybenzene (aminoisophthalic acid). The toxin binding

25   moiety is any molecule that binds to a toxic substance
with high affinity. Examples of such binding molecules
include monoclonal antibodies (or fragments or geneti-
cally engineered counterparts), aptamers, peptides,
oligodeoxynucleoside (or binding fragments), intercala-

30   tion reagents (e.g. dyes, chemotherapy agents, natural
substances), and metal chelates that specifically bind
with the toxin or an effector molecule attached to the
toxic material. Examples of toxic materials include:

metal ions, chemotherapy agents, free radionuclides, radionuclides bound to other compounds, ingested toxins, toxins produced by bacteria, toxins produced by viral infections, toxins produced by disease states, diseased

5　cells, cells involved in the immune response, blood group or HLA incompatibility as well as incompatibility with xenoantibodies.etc.
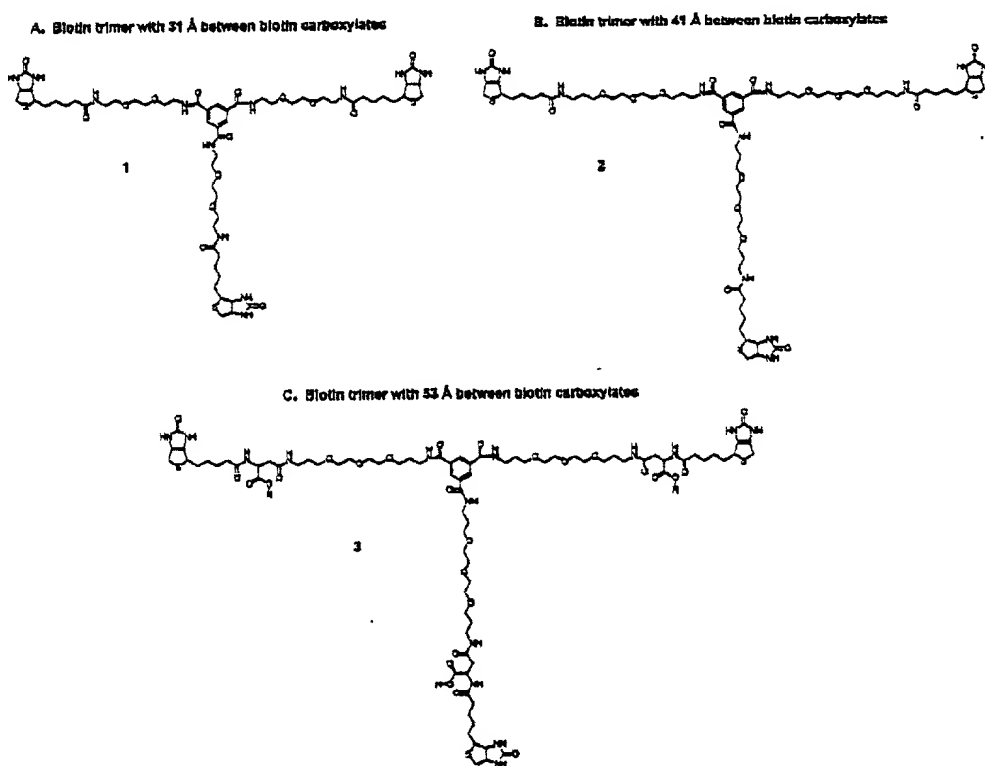
The preferred biotin residue is biotin or a derivative thereof. In most examples the biotin moiety will be

10　natural biotin , which is coupled to a linker through an amide bond. In some examples it may be advantageous to have a biotin derivative that does not bind as tightly as natural biotin, or a biotin derivative that binds to chemically modified, or genetically mutated, avidin or

15　streptavidin in preference to natural biotin. Example of such biotins are norbiotin, homobiotin, oxybiotin, imino-biotin, desthiobiotin, diaminobiotin, biotin sulfoxide, and biotin sulfone. Other modifications of biotin, in-cluding further modification of the above examples are

20　also included. The two biotin moieties in the reagent according to the present invention may alternatively be comprised of any of the biotin derivatives listed above.

In a preferred embodiment of the invention, the toxin binding moiety is biotin or a biotin derivative.

25　Conditioning of the avidin or streptavidin column with this reagent (tribiotin reagent) permits removal of toxic materials that are combined with avidin or streptavidin (or these reagents which are chemically modified, trun-cated by enzymatic digestion, or altered through genetic

30　mutation methods). Examples of a preferred conditioning reagent that contains 3 biotins is shown in Scheme 1. A preferred medical application for this conditioning rea-gent is to remove from a patient's blood, avidin or

streptavidin that has a therapeutic radionuclide bound to
it. In the examples shown (compounds 1-3) the condition-
ing are the same except for the nature and length of the
linker group. The cross-linking reagent employed is

5   1,3,5-benzene tricarboxylic acid, and the linkers used
contain ether functionalities for water solubilization.
In compound 3, the linker also contains aspartic acid
which provides a free carboxylate to aid in water
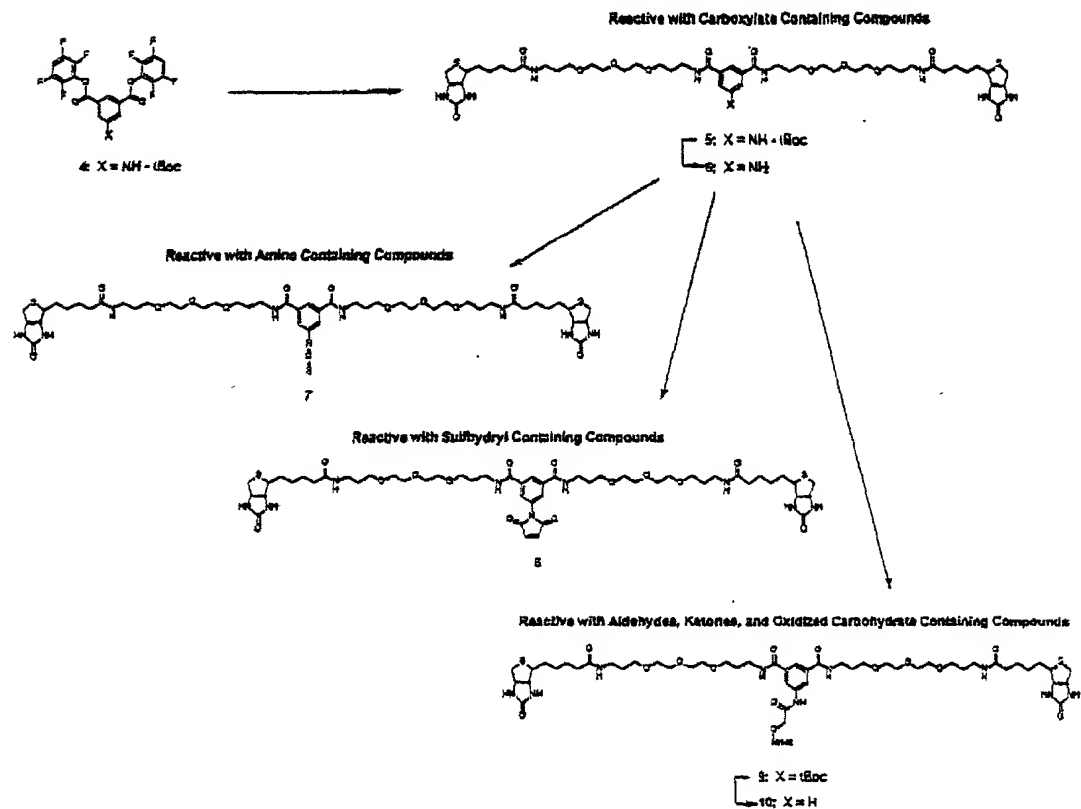solubilization and to block the action of biotinidase.

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Scheme 1: Examples of biotin dimers that also contain a third biotin for binding radiolabeled streptavidin derivatives

A. Biotin trimer with 31 Å between biotin carboxylates

B. Biotin trimer with 41 Å between biotin carboxylates



C. Biotin trimer with 53 Å between biotin carboxylates



Other toxic material binding dibiotin conditioning
15   reagents involved in the methods according to the present
invention can be readily prepared by conjugating a
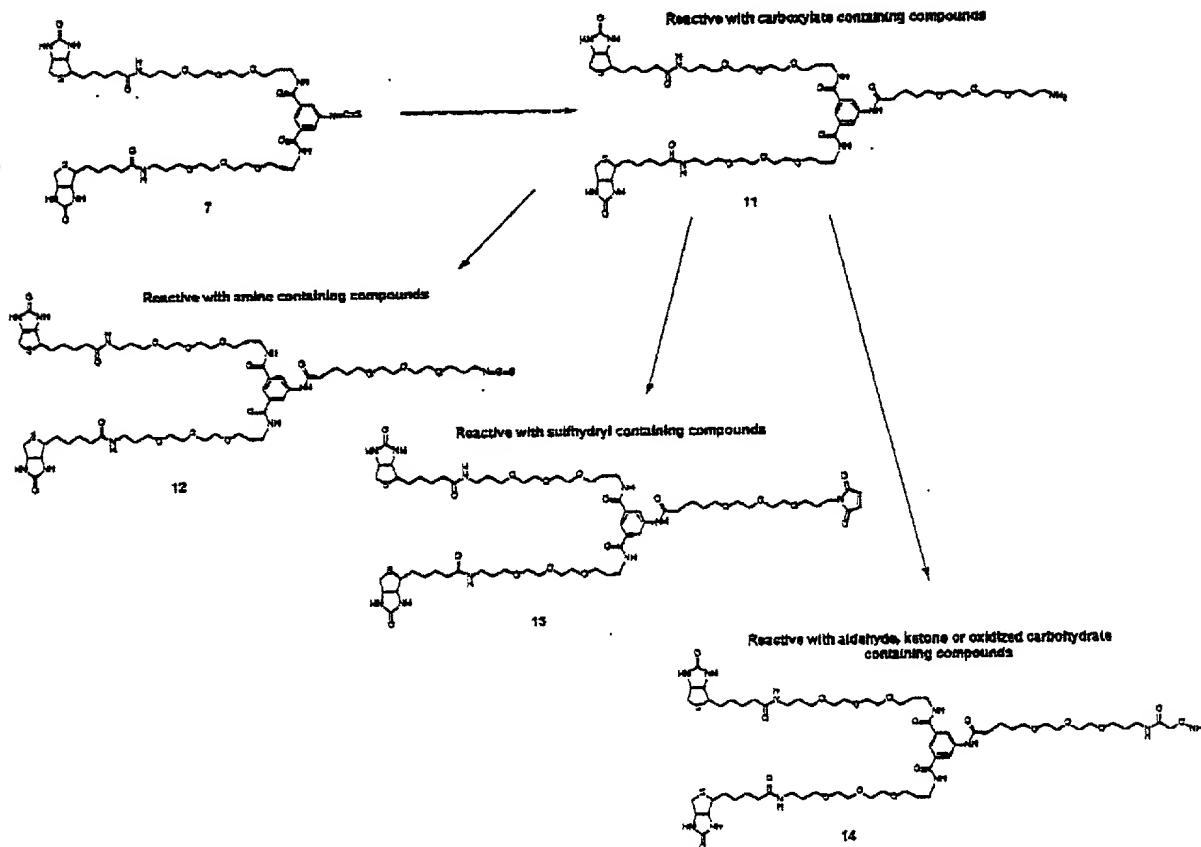nucleophile containing, or nucleophile reactive, dibiotin

compound with the toxic material binding moiety. Examples
showing the synthesis of reactive dibiotin compounds are
provided in Schemes 2 and 3. The tetrafluorophenyl ester
activated and N-tBoc protected aminoisophthalate, 4, is
5    used as the trifunctional cross-linking reagent in these
examples. Reaction of 4 with biotin-conjugated to the
linker, 4,7,10-trioxatridecanediamine provides dibiotin
compound 5. The N-tBoc protection group in 5 is readily
converted to the free amine, 6, with neat trifluoroacetic
10   acid. Anilino compound 6 is reactive with toxic material
binding compounds that contain activated carboxylate
esters or that will undergo other nucleophilic
displacement reactions. The free amine of 6 is also
readily converted to functional groups that are reactive
15   with nucleophiles (e.g. isothiocyanate, 7; maleimide, 8,
or other reagents such as alpha-halo acetamides). The
isothiocyanato-dibiotin compound 7 is reactive with toxic
material binding molecules that contain amines, and the
maleimido-dibiotin 8 is reactive with toxic material
20   binding molecules that contain sulfhydryls (also amines).
Additional reactive reagents can be readily prepared in
the same manner. A reagent that is reactive with oxidized
sugars and alcohols is the hydroxylamine derivative 10.
In the examples in Scheme 2, the linker c is either not
25   present or is first attached to the toxic material
binding molecule. In many examples, a linking molecule
will be desired to make the toxic material binding moiety
more available for interaction with the toxic material in
blood. Therefore, a linker may be built into the molecule
30   prior to reaction with the toxic material binding
molecule. Examples where a linker molecule has been
incorporated are shown in Scheme 3 (compounds 11-14)

**Scheme 2: Synthesis of dibiotin reagents that can be conjugated with other molecules**

**Reactive with Carboxylate Containing Compounds**



4: X = NH - tBoc

5: X = NH - tBoc
6: X = NH2

**Reactive with Amine Containing Compounds**



7

**Reactive with Sulfhydryl Containing Compounds**



8

**Reactive with Aldehydes, Ketones, and Oxidized Carbohydrate Containing Compounds**



9: X = tBoc
10: X = H

21

**Scheme 3: Synthesis of dibiotin reagents which contain a linker moiety and have functional groups which permit conjugation with other molecules**



Reactive with carboxylate containing compounds

7

11

Reactive with amine containing compounds

12

Reactive with sulfhydryl containing compounds

13

Reactive with aldehyde, ketone or oxidized carbohydrate containing compounds

14

## Examples

The following examples are provided to show methods

5    for obtaining various types of compounds disclosed in

this patent and their use as a reagent in conditioning

the column for toxin removal from whole blood. The exam-

ples are provided by way of illustration, and not by way

of limitation.  Many further examples can be envisioned

10   from the examples shown here.

## Example 1

Preparation of a dibiotin compound that can be con-

jugated with toxin binding molecules

Step 1: Preparation of N-tert-Boc-5-aminoisophthalic acid
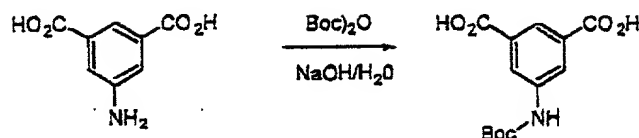


Di-tert-butyl dicarbonate (1.27 g, 5.80 mmol) was

5 added to a solution of 5-aminoisophthalic acid (1.0 g,

5.52 mmol), sodium hydroxide (0.49 g, 12.14 mmol), DMF

(10 mL) and water (10 mL) at ice/water bath temperature.

The reaction mixture was stirred at ambient temperature

for 16 h. The solution was neutralized with 53.0 mL of

10 0.5 N HCl at ice/water bath temperature, then the white

precipitate was filtered, washed with water, and dried

under vacuum. The residue was crystallized from MeOH/$H_2O$

to give the pure compound as a white solid. Yield 0.94 g

(61%). mp >300°C. 1H NMR (DMSO-d6): δ 1.50 (s, 9H), 8.08

15 (t, J = 1.5 Hz, 1H), 8.31 (d, J = 1.5 Hz, 2H), 9.80 (s,

1H). HRMS calcd.

lcd for $C_{13}H_{16}NO_6$ (M + H)+: 282.0977. Found: 282.0981.

HPLC: tR =11.3 min.


20 Step 2: Preparation of N-tert-Boc-5-aminoisophthalate di-
tetrafluorophenyl esther.
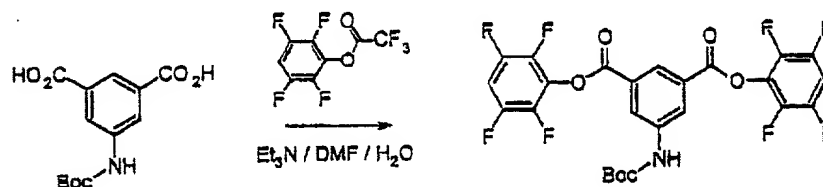


25 2,3,5,6-Tetrafluorophenyl trifluoroacetate (0.47 mL,

2.71 mmol) was added dropwise to a solution of tert-Boc-

5-aminoisophthalic acid (0.35 g, 1.23 mmol), $NEt_3$

(0.52 mL, 3.70 mmol), DMF (4.0 mL) and water (10 mL) at room temperature. A 60 mL quantity of water was added after the reaction mixture was stirred at room temperature for 30 min, the white precipitate was filtered,

5   washed with water, dried in vacuo to give the crude product. The crude product was purified by silica gel column chromatography (40 g) eluting with 10% EtOAc/-hexane to give a colorless solid. Yield 0.213 g (30%). mp 159.7-161.8°C dec. 1H NMR (CDC13):
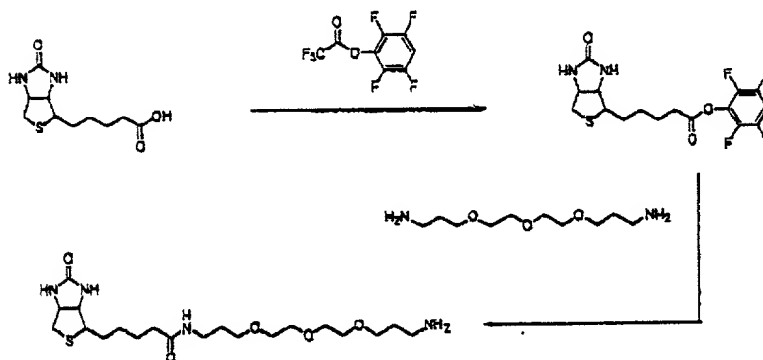
10   1.55 (s, 9H), 6.83 (s, 1H), 7.08 (m, 1H), 8.54 (d, J = 1.5 Hz, 2H), 8.67 (t, J = 1.5 Hz, 1H). HRMS calcd for $C_{25}H_{15}F_8NNaO_6$ (M + Na)+: 600.0669. Found: 600.0674. HPLC: tR = 16.1 min.

15

Step 3:   Preparation of N-(13-amino-4,7,10-trioxa-tridecanyl)biotinamide.



20

Biotin (10 g, 40.9 mmol) was dissolved in 200 mL warm (70°C) DMF under argon atmosphere. The solution was allowed to cool to ambient temperature, 10 mL (82 mmol) triethylamine was added, followed by the addition of 16 g

25   (61 mmol) 2,3,5,6-tetrafluorophenyl trifluoroacetate. The reaction was stirred at room temperature for 30 min

and solvent was removed under vacuum. The product was triturated in 100 mL ether and was filtered. The isolated product was dried under vacuum to yield 14 g (83%) of biotin TFP ester as a colorless solid, mp 185-187°C. 1H

5 NMR (DMSO-d6, ()): 1.4-1.8 (m, 6H), 2.5 (m, 1H), 2.6-2.9 (m, 3H), 3.1 (m, 1H), 4.2 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1 H); IR (KBr, cm-1) 3250, 2915, 1790, 1710, 1520, 1480, 1090. Analysis calc. for $C_{16}H_{16}F_4N_2O_3S$: C, 48.98; H, 4.11; N, 7.14. Found: C, 48.90; H, 4.14; N, 6.86.

10 Biotin TFP ester (5 g, 12.8 mmol) was added to a dry flask containing 200 mL anhydrous DMF. In another dry flask containing 28 g (128 mmol) 4,7,10-trioxa-1,13-tridecanediamine, 8, was added 4 mL of triethylamine. Both flasks were cooled to 0-5°C with ice-water baths.

15 The TFP ester of biotin was added dropwise to the tri-oxatridecanediamine solution over the period of 1 h. The reaction was stirred at room temperature for 30 min and the solvent was removed under vacuum. The resulting oil was triturated in 500 mL ether and was stirred for 30

20 min. The solid was filtered, then dissolved in methanol: ethyl acetate (4:1), and loaded onto a silica column (2.5 cm x 35 cm). The column was eluted with the same solvent mixture. Fractions containing product were collected, and solvent was removed under vacuum. The

25 isolated product was dried under vacuum to yield 4.5 g (79%) of, 9, as a colorless solid, mp 104-106°C. 1H NMR (MeOH, ()): 1.46 (m, 2H), 1.6-1.8 (m, 9H), 2.2 (t, 2H), 2.7 (d, 1H), 2.75 - 2.9 (m, 3H), 3.2-3.3 (m, 5H), 3.5-3.6 (m, 14H), 4.3 (m, 1H), 4.5 (m, 1H); IR (KBr, cm-1):

30 3280, 2910, 2850, 1690, 1640, 1110, 940. Analysis calc. for $C_{20}H_{38}N_4O_5S \cdot H_2O$: C, 51.70; H, 8.68; N, 12.06. Found: C, 51.95; H, 7.98; N, 11.65.

25

Step 4: 1-N-tert-Boc-3,5-Bis(13´-(biotinamidyl)-
4´,7´,10´-trioxatridecanamidyl)-aminoisophthalate.



5          3
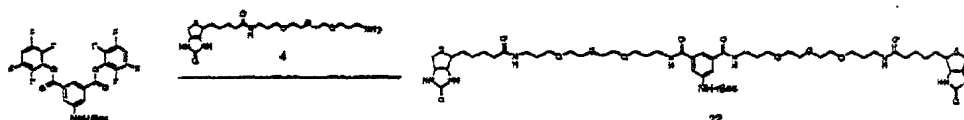
Biotin-trioxadiamine, 4 (100 mg, 0.22 mmol) in
anhydrous DMF was added dropwise to a solution of 3
(65 mg, 0.11 mmol) and triethylamine (47 µL, 0.33 mmol)
10  in anhydrous DMF at rt (room temperature). The reaction
mixture was stirred at rt for 2 h, and then the solution
was evaporated to dryness under vacuum. The residue was
purified by silica gel column (40 g) eluting with 20%
MeOH/EtOAc to yield 73 mg (58%) of a colorless solid, mp
15  209-211°C dec. 1H NMR (CD$_3$OD, 200 MHz): δ1.43 (t, 3H),
1.54 (s, 9 H), 1.69 (m, 6H), 1.88 (m, 3H), 2.19 (m, 4H),
2.69 (d, 4H), 2.92 (m, 2H), 4.30 (m, 2H), 4.48 (m, 2H),
7.83 (m, 1H), 8.00 (m, 2H); mass calcd for C$_{53}$H$_{88}$N$_9$O$_{14}$S$_2$
(M + H))+: 1139.  Found: 1139. mass calcd for
20  C$_{53}$H$_{87}$N$_9$O$_{14}$S$_2$Na (M + Na)+: 1161. Found: 1161. HPLC
11.8 min.

Step 5: 1-Isothiocyanato-3,5-Bis(13´-(biotinamidyl)-
4´,7´,10´-trioxatridecandyl)-aminoiso-phthalate
25

26
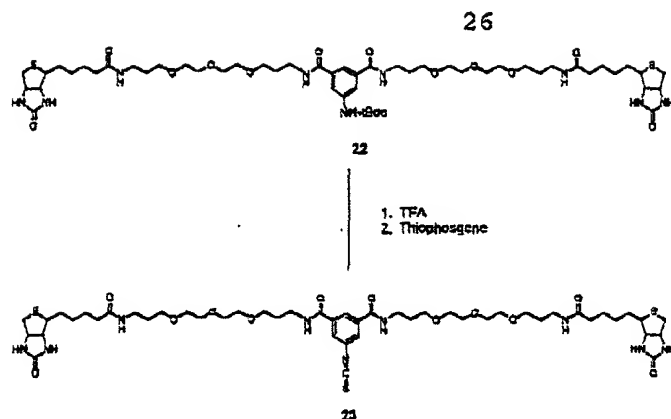


A 120 mg quantity of 22 (0.11 mmol) was dissolved in
neat TFA (1 mL) and stirred at rt for 10 min. Following
this, excess TFA was removed under vacuum. The residue
was dissolved in 2 mL of methanol and treated with 0.2 mL
of triethylamine. The volatile materials were removed
under vacuum, then water (3 mL), chloroform (3 mL) and
thiophosgene (42 $\mu$L, 0.55 mmol) were added respectively.
The mixture was stirred at rt for 1 h. Following this,
excess thiophosgene and chloroform were evaporated in
fume hood under a stream of argon. The remaining aqueous
phase was evaporated to dryness under vacuum to afford 77
mg (68%) of 23 as a light yellow tacky solid. 1H NMR
(DMSO-d6, 200 MHz): $\delta$ 1.24-1.35 (m, 6H), 1.43-1.67 (m,
14H), 1.77 (t, J = 6.6 Hz, 6H), 2.05 (t, J = 7.1 Hz, 6H),
2.58 (d, J = 12.5 Hz, 2H), 2.82 (dd, J = 4.8, 12.5 Hz,
2H), 3.07 (m, 8H), 3.28-3.57 (m, 18H), 4.13 (dd, J = 4.6,
7.7 Hz, 2H), 4.31 (dd, J = 4.6, 7.7 Hz, 2H), 7.80 (t, J =
5.0 Hz, 2H), 7.98 (s, 2H), 8.34 (s, 1H), 8.77 (t, J = 5.1
Hz, 2H) mass calcd for $C_{49}H_{78}N_9O_{12}S_3Na$ (M + Na)+: 1103.
Found: 1103. HPLC 11.8 min.

Example 2

Conjugation of a dibiotin compound with a toxin binding
molecule

A 108 mg (15 equiv.) quantity of the dibiotin
isothiocyanate compound, 23, in 4 μL DMSO was added to
150 μL of a 6.7 mg/mL solution of the monoclonal antibody
5    53-6A2 (1 mg). The mixture was lightly vortexed, then
allowed to react at room temperature overnight. The di-
biotin conjugated antibody was purified from excess di-
biotin reagent by ultracentrifugation filtration at
6000 rpm in a Centricon 30, followed by 4 x 1 mL washings
10   with 0.9% saline.

Example 3

Conditioning of an avidin column with a dibiotin -toxin
binding conjugate

      This example provided is that of a dibiotin compound
15   that also contains another biotin moiety. Two of the bio-
tin moieties will bind with avidin or streptavidin leav-
ing the third biotin moiety available for binding with
toxic compounds that are also conjugated with, or are
fusion proteins containing, avidin or streptavidin. Thus,
20   in this example the dibiotin compound is a biotin trimer,
but the same methods may be used to condition a column
with dibiotin compounds that contain other high affinity
binding ligands.

      Conditioning of a biotin binding column to convert
25   it to an avidin-binding column:

      Two mL of Mitra Avidin-Agarose was packed into a
column housing and was washed with >10 mL of PBS at a
flow rate of 1 mL/min (1.6 cm/min). Five mL of a 1 mg/mL
solution of biotin-trimer in PBS was recirculated through
30   the avidin-column at 1 mL / min for 20 minutes. At end of
the recirculation, a sample (0.5 mL) was drawn from the
recirculated solution and analysed by an ELISA technique
developed by Mitra Medical Technology AB. The biotin-

avidin-agarose-column was washed with phosphate buffer
at 1 mL / min for 20 minutes. By determining the concen-
tration of biotin-trimer in the recirculated fluid, the
amount of adsorbed biotin-trimer was estimated to about
5   1.9 mg i.e. 0.95 mg/mL of gel at the end of recircula-
tion.  The standard curve for determining the concentra-
tion of biotin-trimer is shown in Fig.4.

Biotin-trimer was adsorbed to 2 mL of Mitra Avidin-
Agarose. The batch of avidin-agarose utilized had a
10  static binding capacity of biotin of about 74 g/mL. If
one biotin-trimer is bound per available binding site
this would correspond to 514 g biotin-trimer/mL. As about
0.95 mg biotin-trimer was bound per mL avidin-agarose it
can be assumed that the adsorbent was saturated with
15  biotin-trimer.

Assessment of avidin adsorption on a conditioned
column:

An avidin-agarose column that was conditioned with a
biotin trimer (i.e. Figure 4, compound #2) was primed
20  with phosphate buffer. Following this, 20 mL avidin-
solution with 1 mg avidin / mL in PBS was recirculated
through a biotin-avidin-agarose-column at 1.0 mL / min
(1.6 cm/min). Three volumes of the avidin solution (3 x
20 mL) were processed and aliquots were drawn from the
25  reservoir before start of the recirculation, then after 2
min and in 5 min intervals. The aliquots were analyzed
for the quantity of avidin in them using an ELISA
technique developed by Mitra Medical Technology AB. The
concentration of avidin in the recirculation reservoir is
30  shown in Fig. 5. The dotted line represented the theo-
retically expected if no saturation effect has occurred.
The column is saturated after about 40 min (2 vol.) when
about 16 mg (80%) of avidin has been bound. Signs of

saturation was seen after about 20 min (1.0 vol.) when the experimental curve begin to deviate from the theoretical curve which occurred when about 10 mg of Avidin had been bound to the column.

5      After a 40 min recirculation, about 16 mg of avidin was bound, i.e. 8 mg/mL biotin-avidin-agarose. This corresponds to a 1:1 molar ration between the avidin coupled to agarose and the avidin adsorbed to the biotin-trimer. Initial saturation effects were seen after about
10    one recirculated volume. After 2 volumes processed no further avidin was bound.

The experiments showed that the Avidin-Agarose can be saturated with a biotin-trimer, binding to all sites available for monomeric biotin and that recirculated free
15    avidin efficiently binds to a column packed with the biotin-avidin-agarose. About 8 mg avidin was bound per mL of adsorbent, corresponding to a 1:1 molar ratio between bound avidin and avidin immobilized to the agarose particles.

20    Example 4

Use of a conditioned column to remove toxins from blood

To facilitate the removal of specific toxins from the blood circulation by passing the blood through an avidin/streptavidin coated device which has prior to the
25    use been converted to a specific device by passing a solution containing a column conditioning reagent carrying a specific toxin binding moiety. This would allow the avidin/streptavidin coated device to be used as a technology platform for the removal of various toxins. In spe-
30    cific cases it could be desirable to remove more than one toxin in the same treatment procedure which could easily be achieved by passing a mixture of column conditioning reagents carrying different specific toxin binding

moiety. A suitable application for such a multifunctional
device could be in the blood clearance of anti-HLA anti-
bodies, anti-blood group antibodies or anti-xenoantibod-
ies prior to organ or cell transplantation. By using a

5    suitable mixture of column conditioning reagents carrying
different specific toxin binding moiety directed towards
specific sub-types of e.g. anti-HLA antibodies, the toxin
removal device can be tailor-made for the patients need
prior to treatment.

10        The conditioning can occur in a the hospital by
connecting infusion bags containing the appropriate
conditioning reagents carrying different specific toxin
binding moieties directed towards specific sub-types to
the monitoring unit (reprogrammed dialysis machine) used

15    in the extracorporeal treatment, and the conditioning of
the avidin/streptavidin device could be achieved manually
or automatically by the monitoring unit. The flow rate of
the column conditioning reagent solutions entering the
device will in such case determine the proportion of the

20    different specific toxin binding moieties in the final
conditioned device.

        Alternatively, infusion bags containing mixtures of
column conditioning reagents carrying different specific
toxin binding moieties or the final devices could be pre-

25    manufactured with certain mixture of specific toxin bind-
ing moieties.

Example 5

Application of a conditioned column to improve immuno-
targeting in a two step procedure

30        To improve immunotargeting in a two-step procedure
by in the first step providing means of efficiently
clearing the blood circulation from biotinylated tar-
geting molecules by passing the blood through biotin-

binding device and in a second step clearing the blood
from subsequently administered toxic derivative of
avidin/streptavidin by passing the blood through an
avidin/streptavidin binding device which has been pro-
5    duced by passing a solution containing the reagent
according to the present invention, e.g. biotin dimers or
trimers, through the biotin-binding device.

Alternatively, the order of this process can be
reversed as follows.

10   To improve immunotargeting in a two-step procedure
by in the first step providing means of efficiently
clearing the blood circulation from SA/avidin conjugated
targeting molecules by passing blood through an avidin/-
streptavidin binding device which has been produced by
15   passing a solution containing biotin dimers or trimers
through the biotin-binding device, and in a second step
clearing the blood from molecules containing the reagent
involved in the methods according to the present inven-
tion, e.g. biotin and a radionuclide/ cytotoxic moiety by
20   passing the blood through a biotin binding device.

Yet another alternative is to improve immunotarget-
ing in a two-step procedure by in the first step provide
means of monitoring the tumor up-take of an biotinylated
targeting molecule labeled with an agent which can be
25   detected by a gamma-camera, PET-scan, MRI or other in
vivo diagnostic techniques and after appropriate time,
clear the blood from non-target bound targeting molecule
by passing the blood through an biotin-binding device and
at appropriate time administer avidin/SA carrying an cell
30   killing radionuclide/ cytotoxic agent which is later
cleared from the blood circulation by passing the blood
through an avidin/streptavidin binding device which has
been produced by passing a solution containing the

reagent according to the present invention, e.g. biotin dimers or trimers through the biotin-binding device.

Example 6

Application of a conditioned column to improve immuno-
5  targeting in a three step procedure

To improve immunotargeting in a three-step procedure by in the first step providing means of efficiently clearing the blood circulation from biotinylated target-ing molecules by passing the blood through an biotin-
10  binding device and in a second step clearing the blood from administered avidin/SA, by passing the blood through an avidin/streptavidin binding device which has been pro-duced by passing a solution containing the reagent according to the present invention, e.g. biotin dimers or
15  trimers through the biotin-binding device and in a third step clearing the blood from molecules containing biotin and a radionuclide/ cytotoxic moiety by passing the blood through a biotin-binding device.

20